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The ryanodine receptor: advances in structure and organization

Alan J Williams, N Lowri Thomas and Christopher H George

Ryanodine receptor Ca^{2+} release channels (RyR) are the largest intracellular ion channels known. The primary physiological role of the RyR is to provide a ligand-regulated pathway for the release of stored Ca^{2+} to regulate a diverse array of cellular processes. This article highlights advances in RyR channel structure, shedding new light on closed-to-open channel transitions and on the mechanisms of channel gating in health and disease. We also evaluate how recent studies on the self-association of RyR in situ have revealed a potential relationship between the dynamic organization of channels in clusters and the termination of the regenerative feedback of Ca^{2+}-induced Ca^{2+} release. Lastly, we consider how this information might impact on the development of new RyR-focused therapies.

Address
Molecular Cardiology, Institute of Life Sciences, Swansea University Medical School, Singleton Park Campus, Swansea SA2 8PP, UK

Corresponding author: Williams, Alan J (alan.j.williams@swansea.ac.uk)

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Introduction

Ryanodine receptors (RyR) are intracellular ion channels that act as conduits for the regulated release of stored Ca^{2+} to affect diverse cellular processes. Initially identified as 'feet' structures in the sarcoplasmic reticulum (SR) of native tissue [1,2], the molecular characterization of the channel was facilitated by virtue of its interaction with the insecticidal plant alkaloid ryanodine. A tetrameric arrangement of ~560 kDa subunits results in a channel of ~2.2 MDa making RyR the largest ion channel structure identified to date. Three mammalian isoforms (RyR1-3) are expressed from different genes that exhibit a high level of exonic organization and encode homologous proteins that share common structural motifs/elements (Figure 1). RyR1, 2 and 3 vary in their tissue distribution; RyR1 and 2 mediate the release of Ca^{2+} that binds to and activates the contractile machinery during skeletal and cardiac muscle contraction, respectively. RyR2 is also expressed at low levels in mammalian brain. RyR3 is thought to play a contributory role to modulating Ca^{2+} signalling in smooth and non-muscle cell types and in excitable tissue (e.g. brain). The three isoforms are also characterized by different mechanisms of activation with RyR1 gated primarily by mechanical interaction with the sarcolemmal voltage-gated Ca^{2+} channel whereas RyR2 and RyR3 are ligand-activated, mainly by cytosolic Ca^{2+}. The efficacy and precise mechanisms for ligand activation of RyR2 and RyR3 are different.

This review focusses on how recent advances in the understanding of RyR channel structure, gating and co-operativity can be used to provide insight into key aspects of the mechanisms of channel function. We will consider how this new information can be reconciled with normal RyR function and with channel dysfunction associated with a wide range of skeletal and cardiac diseases (e.g. arrhythmias provoked by chronic abnormalities in RyR2 channel phosphorylation or occurring as a consequence of genetic mutations that sensitize the channel to activating ligand).

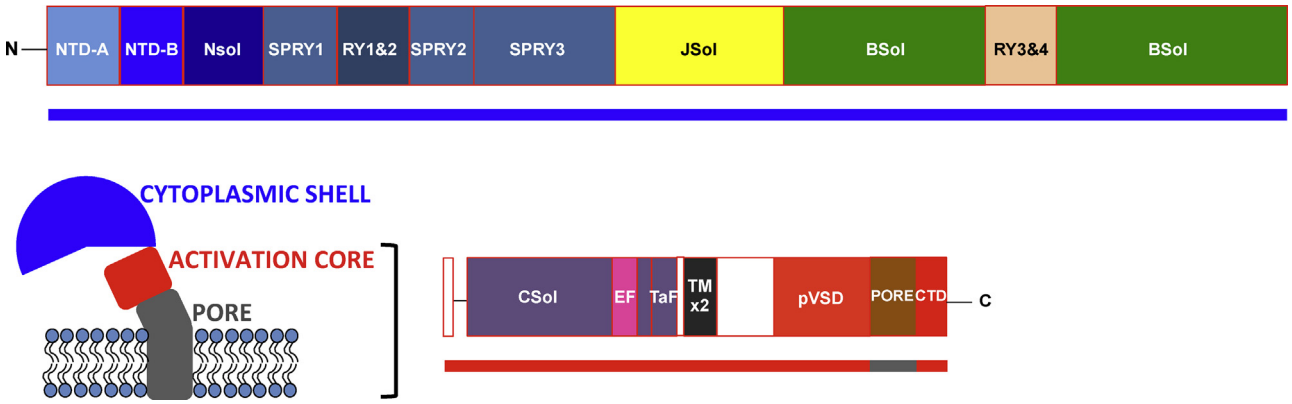
New advances in RyR structure

The enormous size of the RyR channel, and the practical limitations in generating material in sufficient quantities required for crystallographic studies, mean that progress in the determination of RyR structure has been comparatively slow. In the absence of crystallographic information, RyR tetrameric structure has been investigated using cryo-electron microscopy (cryo-EM), with early, low-resolution, structures emerging since the late 1980s [3,4]. Incremental technical improvements have increased resolution so that structural information on RyR1 and, to a lesser extent RyR2, is now approaching atomic resolution. In 2015 three groups published high-resolution structures of closed conformations of RyR1 [5–7] and these studies have recently been comprehensively and critically reviewed [8••].

The physiological role of RyR2 in cardiac muscle is to provide a ligand-regulated pathway for the release of stored Ca^{2+} to initiate contraction, and disruption of regulation results in disease and sudden death. As a consequence, the processes underlying the conformational rearrangements between closed and open states of RyR channels are of particular interest and in 2016 four groups published high-resolution cryo-EM investigations of the structural basis for gating in RyR1 [9••,10••,11••] and RyR2 [12••].

There is broad agreement on the structural domains that make up the RyR monomer (Figure 1) and the arrangement of these monomers in the tetrameric channel.

Figure 1



Two dimensional representation of domain boundaries of the RyR1 monomer described in des Georges et al. [9••]. The solid lines beneath the layout correspond to the three structural elements depicted in the cartoon illustration of the RyR monomer which is based on the schema of des Georges et al. [9••]. Definitions of the abbreviated names of domains, and details of the residues included in each domain can be found in des Georges et al. [9••]. Equivalent domain boundaries and names used in the other investigations discussed in this article can be found in Bai et al. [10••], Wei et al. [11••] and Peng et al. [12••]. This cartoon structure is used in Figure 2 to provide an overview of the closed and open RyR conformations observed in the studies discussed in this article.

Higher resolution attained in des Georges et al. [9••] permitted the identification of distinct elements within some of these domains. Different methods have been used to capture open and closed RyR conformations and these are summarized in Figure 2 and in the annotated references. Information on the widest range of potential conformations was obtained by des Georges et al. [9••] and we will use this study as a framework within which to discuss the observations of all four investigations. des Georges et al. identify three major structural regions of the RyR1 monomer: the shell, the activation core and the pore (Figure 1). In the absence of activating ligands des Georges et al. [9••] report a number of distinct conformations in which the pore is closed but the position of the shell varies, moving up and down relative to the plane of the SR membrane (Figure 2). Bai et al. [10••] report equivalent movements of the cytoplasmic shell in closed conformations of RyR1, describing this as a 'breathing motion'.

For RyR1 activated by either Ca^{2+} alone or a combination of caffeine and ATP, des Georges et al. [9••] report conformations in which the pore remains closed but the structure of the activation core is altered, and describe these conformations as 'primed' for activation. In 50% of RyR1 structures exposed to a combination of Ca^{2+}, caffeine and ATP the pore was open. In this conformation the cytosolic shell and activation module lean away from the central axis of the tetramer, opening the pore as the result of bending and bowing of the pore-lining S6 helices.

Bai et al. [10••] used a combination of Ca^{2+} and PCB95 to obtain an open conformation of RyR1. Comparison with a closed conformation demonstrated that transition between the states involved structural rearrangement of the channel's central domain (broadly equivalent to the activation core in des Georges et al. [9••]), triggering pore opening as the result of bending of S6.

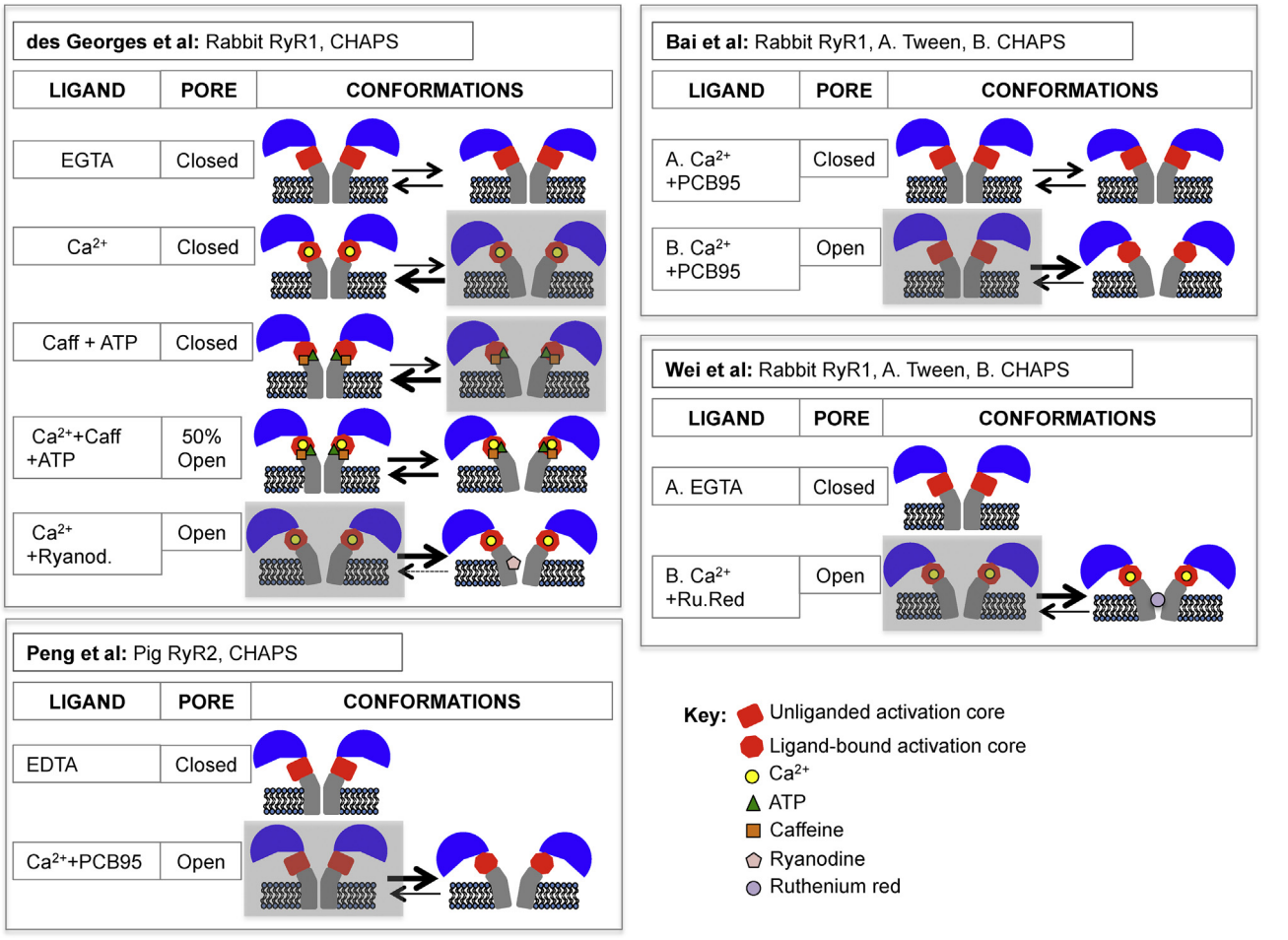
Wei et al. [11••] activated channels with Ca^{2+} followed by incubation with ruthenium red, arguing that under these conditions RyR1 would be stabilized in an open, blocked, conformation. When compared with a closed RyR1 structure [6] the authors describe a 'breathing motion' of the cytosolic shell in the transition, together with an upward movement of the central domain that results in an outward movement of S6 helices.

Closed and open conformations of the RyR2 channel were reported by Peng et al. [12••] respectively in the absence of activating ligands and in the presence of Ca^{2+} and PCB95. Comparison of the resulting structures revealed changes in the cytoplasmic shell and the central domain similar to those observed in RyR1 by des Georges et al. [9••] and Bai et al. [10••]. In RyR2 turning of the central domain causes an outward motion of the cytosolic ends of S6 helices to open the pore.

All closed conformations observed in these studies have a physical barrier to cation translocation created by the close apposition of hydrophobic residues of the pore-lining S6 helices. In both RyR1 [9••,10••,11••] and RyR2 [12••], equivalent isoleucine residues form this cytosolic gate. The deformation of S6 helices in the open conformation widens the pore and removes the barrier to cation flow.

The open conformations reported in these studies are ligand-activated. Both des Georges et al. [9••] and Wei

Figure 2



Overview of RyR conformations. Each panel shows the source material and detergent used to extract RyR from the SR membrane, together with the ligands used to manipulate channel open probability. Details of methods, concentrations, etc., can be found in the source articles. Conformations of RyR are shown using the cartoon structure described in Figure 1. In each case two opposing monomers are used to depict the conformation of the shell, activation core and pore of the channel. Equilibria between potential conformations in each sample are shown. Conformations with a low probability of occurrence, and not observed, are shaded. See text for more detail. For des Georges et al. [9••] and Wei et al. [11••] bound ligands are shown in the activation core (see source articles for details of residues involved). No potential binding sites were identified in Bai et al. [10••] and Peng et al. [12••].

et al. [11••] identify a Ca^{2+} binding site, although not the same site, within the activation/central domain of the channel and des Georges et al. [9••] additionally identify binding sites for ATP and caffeine within the same region of RyR1. Ligand-binding-dependent structural changes are transmitted to both the cytoplasmic shell and the pore to open the channel. The mechanism of activation during E-C coupling is different for RyR1 and RyR2. The trigger for RyR2 opening comes from an increase in cytosolic Ca^{2+}, while RyR1 opening is initiated by a physical link to voltage-dependent structural rearrangements of the sarcolemmal Cav1.1 channel. Bai et al. [10••] and Wei et al. [11••] propose that physiological activation of RyR1 involves an initial conformational alteration of the cytoplasmic shell that is transmitted through the central region to the pore.

These studies have captured open conformations of RyR by using conditions that maximize the likelihood of channel opening. Functional studies of RyR gating have established that a range of open, kinetically distinguishable, states exist with average durations measured in milliseconds [13]. Given their very short duration, these could never be captured in structural studies. However, while other regions of the channel may show structural variation, as these states have the same unitary conductance, it is probable that the structure of the pore in each open state will be very similar. The high-affinity

interaction of ryanodine with RyR stabilizes an open conformation of reduced conductance and des Georges et al. [9••] obtained structural information for ryanodine-modified RyR1. They conclude that ryanodine binds within, and partially occludes, the open pore to reduce conductance. This conclusion contrasts with functional studies that used comparative molecular field analysis to investigate the characteristics of ryanodine analogs that determined modified conductance and indicated that rather than directly obstructing the pore, ryanodine reduces conductance by inducing or stabilizing conformational alterations in the open pore [14].

Clustering of RyR channels: its role in excitation-contraction coupling

It has become increasingly clear that RyR2 channel distribution within the SR is critical in determining the properties of cardiomyocyte Ca^{2+} handling. The Ca^{2+} induced Ca^{2+} release (CICR) mechanism inherent in triggering the release of SR Ca^{2+} required for muscle contraction is a positive feedback process, and yet cannot be endlessly regenerative. This apparent paradox was initially resolved using theoretical modelling [15] in which the channels were positioned into small, functionally isolated groups or clusters, allowing 'cluster bomb activation' to initiate and 'stochastic attrition' to terminate the event. Subsequent observation of spatially isolated Ca^{2+} sparks [16] provided the basis for local control theory [17], which states that Ca^{2+} release does not spread out of control because of the physical separation between RyR2 clusters and because of the high Ca^{2+} concentration required for activation. In recent years, the increasing accessibility of super-resolution optical techniques has revealed that RyR2s do not pack in regular 'checkerboard' arrays (as in skeletal muscle [18]) but rather form an irregular network of channel clusters [19,20••]. Clusters were recently found to be irregularly spaced and composed of different numbers of RyR2s averaging 14–15 [20••,21], much smaller than the 75–100 previously estimated [22]. In silico modelling has shown that the organization and size of clusters is expected to affect both the sensitivity of the channels to activation [23] and the termination of release [19], affecting spark fidelity (i.e. the probability that a spontaneous channel opening triggers a Ca^{2+} spark [24]) and duration [25].

Channel clustering is considered to be the result of allosteric coupling between channels, which is thought to allow simultaneous opening and coordinated closure (known as 'coupled gating') [26,27] and also to keep channels closed at low Ca^{2+} and open at activating Ca^{2+} concentrations [23]. Indeed, coupled RyR2 channels differ in their gating, exhibiting longer open and closed times compared to single channels [26–28]. Recent transmission electron micrographs of RyR2 [29••], show the channels interacting in two configurations: 'adjoining' (side-by-side) and 'oblique' (similar to the 'checkerboard' arrangement of RyR1, but with the channels at a 12° angle to one another, rather than parallel). The oblique interaction is through SPRY1 and P1 domains, and because this is an asymmetric interaction compared to that proposed for RyR1, there is steric hindrance to forming a continuous checkerboard, instead favouring a less ordered 'branched' array, fitting with that which has been observed in cardiac tissue [20••,21,22]. Packing in similar adjoining and oblique formation has also been observed in rat ventricular myocytes in images generated using dual tilt tomography to produce en face views of channels in the SR [30]. This study found these arrangements to be labile, with the two different packing orientations being influenced by physiological conditions with mainly oblique packing observed upon phosphorylation or at low Mg^{2+} concentrations, with higher Mg^{2+} concentrations resulting in more side-by-side or adjoining channel packing. Interestingly, the presence of Mg^{2+} has also been a requirement for the observation of coupled gating in bilayer experiments [28].

However, whether the exact arrangement of channel packing has functional implications for Ca^{2+} release is currently unclear and the phenomenon of 'coupled gating' is controversial. This is mainly due to the lack of direct experimental evidence; even in recordings that appear to evidence simultaneous gating of channels [26,27] these data cannot be conclusively reconciled with the relative spatial arrangements and physical interactions of the multiple channels in the bilayer.

Despite this, it has been suggested that decreased allosteric coupling due to changes in cluster organization plays a role in cardiac disease [23] with variation in cluster distribution being observed in a sheep model of atrial fibrillation [20••], and altered coupled gating of mutant RyR2s proposed to cause delayed after-depolarisations in the genetic arrhythmia syndrome CPVT (catecholaminergic polymorphic ventricular tachycardia) [31]. It has been suggested that smaller or less organized clusters (i.e. ones with gaps in them) are likely to have lower levels of allosteric regulation and so would be likely to fire more readily, contributing more to diastolic release [32]. This is in keeping with the concept of unclustered channels responding differently to local Ca^{2+} concentration [23]. However, as these leaky, 'rogue' channels are not part of a cluster this prevents the formation of a spark, meaning that this Ca^{2+} leak goes undetected by cellular spark imaging methods i.e. sub-spark or quarky Ca^{2+} release [23]. CPVT-linked human RyR2 mutation has been proposed to result in reduced co-operativity of channel gating, and in silico modelling has identified this as a possible substrate for arrhythmia [31,33]. Future studies are anticipated to unveil the packing order (i.e. whether channels are in a side-by-side/adjoining or chequerboard/oblique lattice arrangement), the uniformity of packing

(i.e. complete or if there are gaps) and the influence of cluster shape on RyR2 function.

Implications for RyR-targeted drug design

An important question remains as to how these insights into RyR channel structure and organization can be used to inform mechanism-based approaches to drug design to treat cardiac disease. The long-held view is that the structural and functional complexities of ion channels preclude rational drug design [34]. The pore-forming region of RyR is the site of promiscuous interaction with canonical chemical structures that contain hydrophobic and ionizable nitrogen groups (i.e. most anti-arrhythmics) and to date, the only ligand that exhibits specificity for RyR is ryanodine, which is not clinically useful. Given that conventional anti-arrhythmics may not represent ideal starting points for new RyR-targeted drug design, it is important to note that the emergence of flecainide [35], carvedilol [36] and dantrolene [37,38] as drugs reported to modulate RyR channel function have come from 'reverse translation' (i.e. these are drugs with established clinical benefit in which their mechanism of action has been back-fitted to a direct or indirect inhibitory effect on the RyR channel) rather than having emerged from feed-forward development using RyR structural information. We should comment specifically on the mechanism of action of flecainide, which was initially reported to directly block RyR2 [35]. In subsequent investigations [39], we have shown that block is unlikely to be its mechanism of action; rather it acts via modulation of intracellular Na^+ and Ca^{2+} as a consequence of use dependent block of sarcolemmal Na^+ channels.

There is also the possibility that some newer chemical entities (e.g. S107 [40], DP2114–2149 [41]) may promote the stabilization of defective disease-linked interactions *between* RyRs (i.e. inter-tetrameric) which could influence long-range conformational ordering across clusters.

In summary, we do not believe that the newly elucidated features of RyR structure will facilitate a step-change in developing RyR2-targeted pharmacologies and nor do these studies eliminate the need for the detailed functional assessments of RyR2 channel-drug interaction. The advances in the field considered in this review should mark the beginning of a new phase of investigations in which new structural insights continue to inform an integrated structure-function approach at the molecular, cellular and organ levels and ultimately in vivo.

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